RPE SBIR Grant Report

Geographic atrophy (GA) pathogenesis is still nebulous and there are no FDA-approved therapies for the 1 million people in the United States who already have GA and the millions more who are at risk. Stem cell based therapy holds great promise to halt and possibly repair the degeneration due to GA. Here CalCyte's team wanted to develop a chemically defined differentiation and culture condition which can be directly translate into a GMP procedure for human RPE cell production from autologous iPS cells. This work will lay the ground for autologous engraftment treatment in GA patients.

To accomplish this goal, CalCyte team has already finished the following studies. Here we report the program progress and results.

For Aim 1. Development of methods differentiating human iPS cells into RPE cells in chemically defined condition with defined growth factors/cytokines.

CalCyte team has already developed a N2 and B27 supplements based feeder-free human ES cell culture method before. And we have successfully differentiated human iPS cells into RPE cells in this chemical defined Chem-D medium. So we would just focus on improving the condition of differentiation and test the in vivo and in vitro functionality of the RPE cells produced under this chemical defined condition.

Subaim 1a, Differentiate monolayer human iPS cells into RPE cells with defined cytokines in chemical defined condition.

We cultured the human iPS cells in Chem-D medium under feeder-free condition on Matrigel. After the cell colonies grow up, we replaced the medium with Chem-D basal medium plus 100ng/ml DKK-1, 500 ng/ml LEFTY-A for 20 days. ES cell was re-plated onto poly-D-lysine/laminin/fibronectin coated slides in 100-200 cell clumps. The cells were continually cultured in normal Chem-D Basal Medium. On the day 30, the cells were checked for Mitf and Pax6 expression by RT-PCR and immunocytochemistry. The percentage of the Mitf+/Pax6+ colony number was as an indicator of the successful differentiation of iPS cells into RPE progenitor cells. In this study we found 100% of iPSCs were converted to Mitf+/Pax6 RPE progenitors. We also found adding small molecule LDN-193189 at 0.2μM final concentration (a highly potent BMP receptor inhibitor, has been demonstrated in recent publications to ideally replace the use of Noggin protein) could increase the differentiation efficiency.

Subaim 1b, In vivo and in vitro testing of the RPE cells produced under chemical defined condition.

We first tested the RPE cells in vitro. Phenotype of the pigmented cells, iPSC and derived RPE cells were imaged using transmission, electron and confocal microscopy with and without immunohistochemistry. We observed that the pigmented cells had a polygonal shape and formed "cobble stone"-like sheets of cells. Immunostaining with anti-zonula occludens-1 (ZO-1) confirmed the cells contained tight junctions. Furthermore the electron microscopic analysis determined that iPSC-derived pigmented cells were polarized and had microvilli on their apical side, basal membrane on their basal side, melanin granules and characteristic tight junctions.

We further used Immunohistochemical staining and confocal microscopy with Phalloidin antibody to find F-actin was distributed adjacent to the cell membrane, similar to naturally-developing RPE cells. Additional RPE markers were also analyzed, including the early markers MiTF and Pax6 and the more mature markers cis-retinaldehyde binding protein (CRALBP), Bestrophin and RPE65. All of those tests further confirmed the retinal identity of the pigmented cells.

iPSC-derived pigmented cells were incubated with fluorescent latex beads and analyzed by confocal microscopy to find that they phagocytose the fluorescent beads.

Another well-known characteristic of RPE in culture is that they will form a polarized epithelium and transport water from the subcellular space into the medium, forming "domes." The iPSC derived RPE was analyzed for the asymmetric distribution of Na/K ATPase and this water transporting function. The data was positive too.

Then we wanted to test in vivo RPE cell engrafting. The produced RPE cells were labeled with eGFP using well-established lentiviral methods and transplanted as a cell suspension into the subretinal space of adult RPE 65-mutant mice. One week after transplantation, SDOCT was performed to screen for successful graft positioning and morphology, and absence of complications (e.g. retinal detachment). At the end of the experiment, morphometric analysis was performed to quantify numbers of graft (GFP+) RPE cells (identified by morphology and immunohistochemistry for RPE65, Bestrophin) and host photoreceptor cells (nuclear counts in the ONL).

To summarize all above studies, we believe we have established a method of differentiating human iPS cells into RPE cells in chemically defined condition.

For Aim 2. Development of methods for stepwise differentiation of human iPS cells into RPE cells with small molecules:

Subaim 2a. Replacing defined cytokines with known small molecule antagonists of Wnt and TGF-1 signaling pathway during iPS cell to RPE progenitor cell differentiation.

Because using DKK-1 and LEFTY-A to increase the eye field cell production has been proven by several groups, it is reasonable to predict that small molecule antagonists of Wnt and TGF signaling pathways will be able to function the same way. We collected the following small molecules for Wnt and TGF signaling pathways:

Wnt: IWP-2, IWP-3, IWP-4, XAV939 and Wnt-C59

TGF: A83-01, Dorsomorphine, IDE-1, IDE-2, LDN-193189, SB431542

Then small molecules of one pathway were tested with the presence of the other protein antagonist. The efficiency and timing of producing RPE progenitor cells were used as indicators to judge their efficacy. Medium with both DKK-1 and LEFTY-A was used as positive control, and media with only one of them was used as negative control. Similar to the experiments done in Aim 1a, the cells were checked for Mitf and Pax6 expression by RT-PCR and immunocytochemistry. The percentage of the Mitf+/Pax6+ colony number was as an indicator of the successful differentiation of iPS cells into RPE progenitor cells. In this study we found adding small molecule LDN-193189 (TGF pathway molecule) at 0.2μM final concentration or IWP-3 (Wnt pathway) at 10μM final concentration or Wnt C-59 (Wnt pathway) at 0.1μM final concentration could increase the differentiation efficiency.

Combination of LDN-193189 (0.2μM) and Wnt C-59 (0.1μM) was tested in the same way and found this condition could significant increase the differentiation efficacy. This final optimal differentiation medium was carried to the full mature RPE cell differentiation and the generated RPE cells were tested as described in Aim 1b. The data was positive.

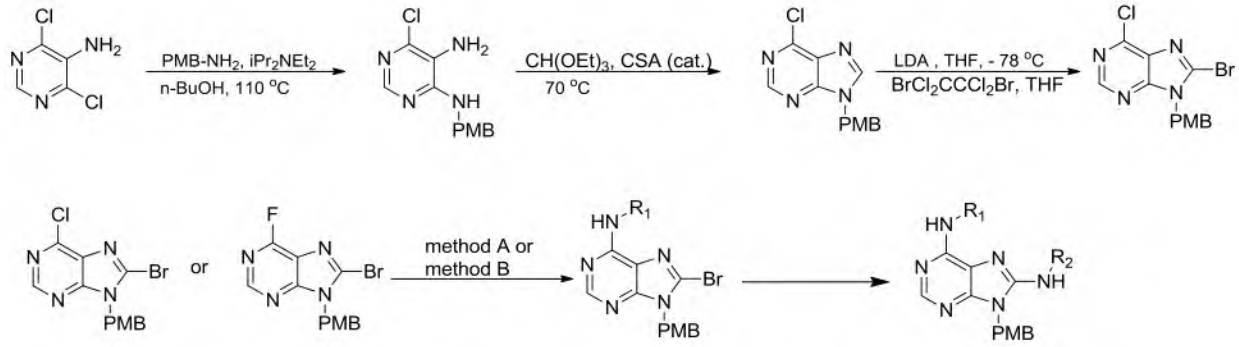
Subaim 2b. Identify novel small molecules that can enhance the eye field cell to RPE cell differentiation.

We used retinal progenitor cells generated from Aim 2a optimal medium culture condition to screen for compounds that can further enhance RPE differentiation/function (using RPE65 and Bestrophin ICC as readouts). We screened the known drug collections (3K drugs) and novel synthetic chemical libraries (50K small molecules) and used the high content imaging platform to automatically analyze the compound-treated cells for identification of initial, pigment-positive hits. Cells en bloc on the poly-D-Lysine/Laminin/Fibronectin coated 384 well plates and small molecules were added. 4 weeks after the plates were fixed and stained for RPE65/Bestrophin/ZO-1 by immunocytochemistry. The cell numbers in each well were detected by DAPI nuclei staining. The small molecules increase the percentage of the PE65/Bestrophin/ZO-1 positive cells were the primary hits. In this screening, we identified 49

primary hits clustered into 5 different scaffolds. Primary hits were confirmed and characterized using a series of secondary assays (expression analysis of RPE cell markers, morphological analysis and functional studies detailed in subaim1b). One scaffold was confirmed to be better than the others based on its physical chemical property, synthesis and further modification possibility and IP space. We further carried out structure-activity-relationships (SAR) study of the selected lead compound to optimize potency and specificity. There were 58 compounds synthesized and tested. The synthesis was summarized below:

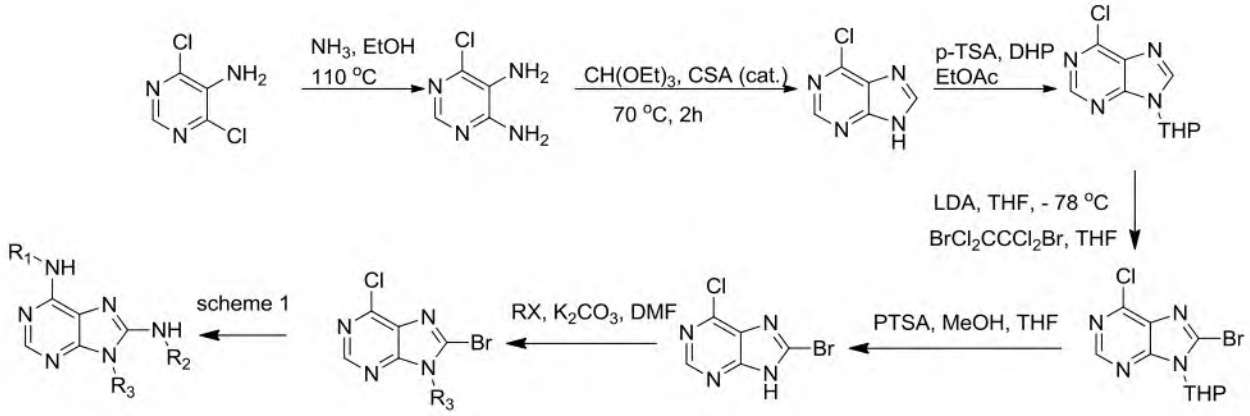
Synthesis scheme:

Scheme 1

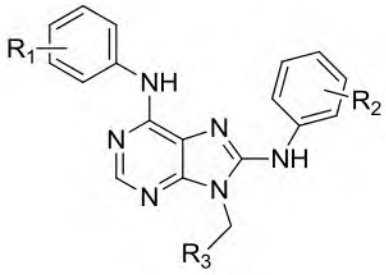


Method A: ArNH2, DIPEA, iPrOH;
Method B: ArNH2, TFA, MeCN or ClCH2CH2Cl

Scheme 2



In this study we identified a lead molecule named CT1055, which improved the efficiency for 400% comparing the no adding this molecules condition.



CT1055

Conclusion:

CalCyte team has finished all of the planed studies for this SBIR grant on time. The team has developed the methods differentiating human iPS cells into RPE cells in chemically defined condition with defined growth factors/cytokines. The team also developed the improved methods for stepwise differentiation of human iPS cells into RPE cells with small molecules. We successfully replaced the defined cytokines with known small molecule antagonists of Wnt and TGF-1 signaling pathway during iPS cell to RPE progenitor cell differentiation. We further identified a novel small molecule CT1055 that can enhance the eye field cells to RPE cell differentiation. Those studies lay ground to develop and apply small molecule driven, stem cell based therapy to treat geographic atrophy (GA) from age-related macular degeneration in the future.